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13. ABSTRACT (Maximum 200 Words)
The overall goal of our study is to develop methods of high-sensitivity and high-specificity for the antemortem diagnosis of prion diseases by detecting PrP_{Sc} in biological fluids using fluorescent immunoassay. During the first year of this contract, we have focused on improving the assay sensitivity by means of new biological protocols and hardware improvement. Concentration of PrP prior to the assay using sodium phosphotungstate treatment was shown to be compatible with the LIFIA and effective in improving the sensitivity. Use of PrP_{Sc}-specific reagents such as antibodies against advanced glycosylation end products (AGE) and DNA provided promising additional approaches for the development of high sensitivity immunoassay. Direct detection of blood PrP_C by Western blot following concentration of PrP by anti-PrP antibody-coated beads improved the potential for detection of PrP_{Sc} in blood. These studies have provided important advancement in our capacity to effectively pursue a specific, sensitive PrP_{Sc} assay in easily obtainable biological fluids and will further our efforts toward the goal of a uorescent immunoassay system using a laser-induced fluorescence spectrofluorometer.

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Introduction

The overall goal of our study is to develop methods of high-sensitivity and high-specificity for the antemortem diagnosis of prion diseases by detecting PrP^{Sc} in biological fluids using fluorescent immunoassay. To achieve our goal, we have focused on: (1) application of various PrP^{Sc} concentration procedures prior to immunoassay, (2) development of immunoassays that do not need proteinase K pre-treatment of samples, (3) detection of PrP^{Sc} in lymph nodes from CWD-affected mule deer and elk, (4) validation of the current immunoassay protocol for the detection of blood PrP, (5) improvement of the sensitivity of the instrument that has been developed for the immunoassay protocols.

Body

1. Magnetic bead-based sandwich immunoassay (designated LIFIA) for PrP^{Sc} coupled with laser-induced fluorescence (LIF) spectrofluorometry.

The existing LIF spectrofluorometer (Rubenstein et al., 2003) equipped with helium-neon laser was used to detect antibodies (Abs) labeled with the fluorescent dye Alexa Fluor 633 (M_r 950; excitation 633 nm/emission 647 nm; $\varepsilon = 159{,}000\ cm^{-1}M^{-1}$). The spectrofluorometer had a limit of detection of ~50 fg/ml of the dye. The fluorescence due to the signal from the sample was corrected by subtracting background fluorescence from the signal and by making a ratio of the sample signal intensity vs. the baseline intensity (S/B ratio). A ratio of 1.10 or lower was determined to be the cutoff value. A ratio above 1.10 was considered positive for PrP^{Sc}.

The basic process for detecting PrP^{Sc} involves the reaction of two Abs specific for PrP: Alexa Fluor 633-labeled anti-PrP 10E4 for detection and biotinylated-4C4 for capture. The biotinylated antibodies were reacted with streptavidin covalently bound magnetic beads (Dynal Biotech, USA). After the series of reactions, the eluted Alexa Fluor 633-labeled antibody complex is measured by LIFIA in 5 mm round glass vials (Kimble/Kontes, USA) with 200 ul volume for 50 sec. A fluorescence reading was obtained by averaging the readings for 10 sec integration times.

2. Application of the LIFIA to the detection of PrP^{Sc} in lymph nodes from Chronic Wasting disease (CWD)-affected mule deer.

Based on the observation that CWD PrP^{Sc} accumulates in alimentary tract-associated lymphoid tissues in early stage of the infection and can be detected with immunohistochemistry before the onset of clinical signs of CWD, it has been suggested that that biopsy material from live deer may be used for an ante-mortem, pre-clinical diagnosis (Sigurdson et al., 1999; Wild et al., 2002; Wolfe et al., 2002). In order to examine the applicability of LIFIA for PrP^{Sc} in extraneural tissues from CWD, retrophrangeal lymph nodes from mule deer and from white-tailed deer were analyzed. One hundred ul of 10% homogenates was used for the assay. Fig. 1 shows the LIFIA of six negative animals and six positive animals. We could detect a specific signal from six positive samples treated with proteinase K (PK), whereas none of the samples from PK-treated negative animals showed a positive signal, yielding a specificity of 100% (Kim et al., 2004). Currently, we are examining more samples to further validate the applicability of LIFIA.

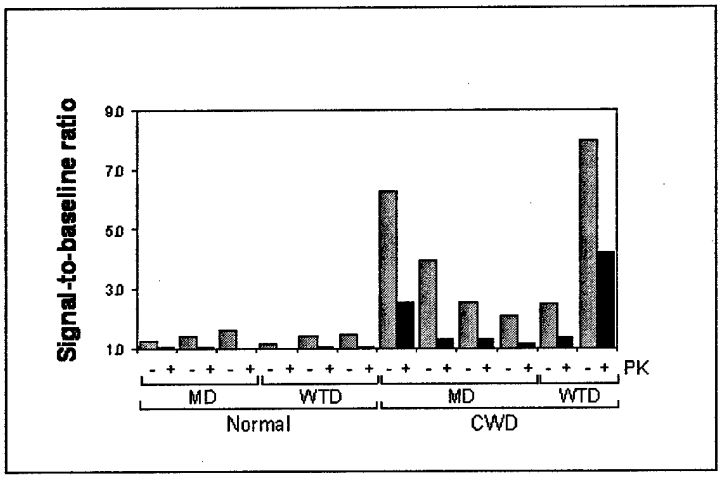

Figure 1. Detection of PrP by LIFIA in retropharyngeal lymph nodes from 6 normal and 6 CWD-affected deer. One hundred ul of normal and CWD-affected lymph nodes from mule deer (MD) and white-tailed deer (WTD) were applied to LIFIA. For PK+ samples, homogenates were treated with 100 ug/ml of PK for 10 min at 37°C prior to LIFIA utilizing Alexa Fluor 633-labled-10E4 and the biotinylated 4C4.

3. Application of various PrP^{Sc}-concentration steps to the LIFIA.

It has been suggested that the successful detection of PrP^{Sc} in biological fluids may be dependent on the ability to concentrate the samples (Wardsworth et al., 2001; Ingrosso et al., 2002); this is important because of the comparatively low level of TSE infectivity outside of the central nervous system (CNS) (Bruce et al., 2001; Hill et al.,

1997; Ramasamy et al., 2003). As an effort to increase the sensitivity of the current immunoassay, application of various concentration steps for PrP^{Sc} to the assay procedure has been tested. PrP-enriched brain preparations from normal and TSE-affected animals were prepared using sodium phosphotungstate (designated NaPTA) or high concentration of NaCl (designated Hi-Salt), as described previously (Wadsworth et al., 2001; Polymenidou et al., 2002, respectively). In addition, 10% brain homogenates in the immunoprecipitation buffer (Tris-Cl, pH 8.0/1% sodium deoxycholate/1% NP-40/150 mM NaCl/1 mM EDTA/ 0.2% SDS) (Kim et al., 2004) were prepared and used for the comparative analysis. Each preparation (equivalent to 2.5 mg of brain tissue) was mixed with immunoprecipitation buffer (300 ul of final volume) containing Alexa Fluor 633-10E4 and biotin-4C4/streptavidin-magnetic beads and then, incubated for 1 hr at room

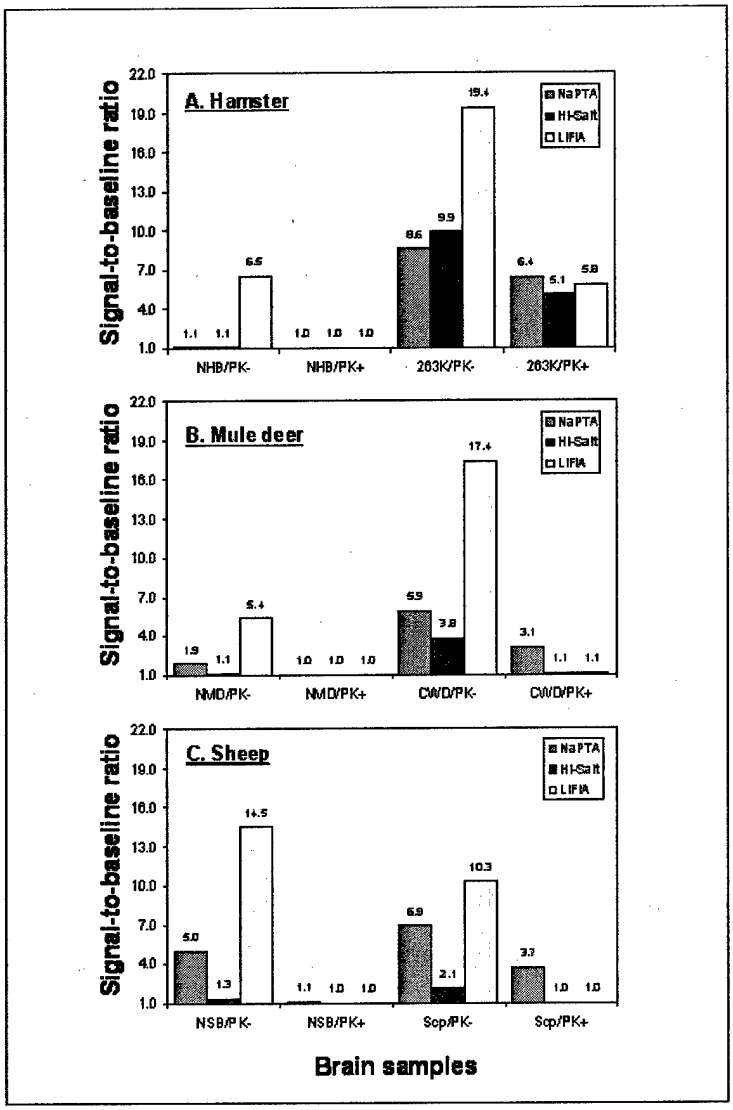

Figure 2. Application of various PrP^{Sc}-enriched preparations to the LIFIA utilizing Alexa Fluor 633-labled-10E4 and the biotinylated 4C4. For PK+, sampes were treated with 100 ug/ml of PK for 30 min at 37°C during the preparation prior to LIFIA

NaPTA; sodium phosphotungstate-treated preparations, Hi-Salt; high concentration of NaCl-treated preparation, LIFIA; 10% brain homogenates in LIFIA immunoprecipitation buffer, NHB; normal hamster, 263K; 263K-infected hamster, NMD; normal mule deer, CWD; mule deer with chronic wasting disease, NSB; normal sheep, Scp; sheep scrapie.

temperature. After washing 3 times with PBS containing 0.1% Tween 20 (PBST), bound material was eluted and fluorescence was measured. As shown in Fig. 2, all non-PK-treated samples from TSE-affected animals showed strong positive signals which varied as a function of the preparation protocol. In the case of PK-treated samples, there were marked differences between preparations. The S/B ratios of Hi-Salt preparations and of LIFIA homogenates were significantly decreased after PK-digestion: this change was most evident with sheep scrapie sample. Although PK-treated CWD samples showed positive signal, the S/B ratio was 1.1 which is just above the cut-off value. However, 263K samples showed fairly high S/B ratios after digestion with PK. The difference in the S/B ration between TSE diseases can be due to the difference between experimental and natural TSE. It is important to note that NaPTA samples were consistently higher in infected compared to uninfected animals, regardless of the species (hamster, mule deer, sheep) and this difference was particularly striking after PK treatment. Taken together, NaPTA preparations were shown to be compatible with the current immunoassay (LIFIA) and will be very advantageous in analyzing natural TSEs.

4. Development of immunoassays excluding proteinase-K treatment.

As an effort to develop more sensitive immunoassays for PrP^{Sc} detection, we are trying to develop the methods to exclude PK-treatment during sample preparation for the assay. Most current biochemical determinations of the protease-resistant PrP^{Sc} rely on the complete removal by PK of the protease-sensitive PrP^{C} so that truncated fragments derived from PrP^{Sc} can be detected by immunological techniques. However, this proteolytic approach can be arbitrary and limits both the specificity and sensitivity of these assays (Barnard et al., 2000; Safar et al., 2002; Kang et al., 2003). Although PK has broad specificity and retains high activity levels in various experimental conditions, the concentration of PrP^{Sc} varies greatly between brain regions as well as between different species. Moreover, conditions that have been optimized for one type of tissue (e.g. brain stem) in the terminal stages of the disease may be inappropriate for the detection of PrP^{Sc} from other tissues (e.g. spleen and blood) or for the detection of pre-clinical disease. In these situations, optimum conditions for proteolysis may need to be constantly readjusted. This approach is further complicated by some studies, which

indicate the detection of infectivity without the presence of PK-resistant PrP^{Sc} (Lasmézas et al., 1997). Furthermore, it has been reported that PK-sensitive PrP^{Sc} is consistently found in brain tissue as well as in the blood of rodents infected with TSE agents (Safar et al., 1998; 2002; Prusiner & Safar, 2000). The identification of an antibody (Ab) that binds selectively to PrP^{Sc} from various species would provide a new means to identify PrP^{Sc} directly without using PK resistance as a criterion.

4.1. Application of antibody against advanced glycation end products (AGE)-modified protein to LIFIA.

Recently, using rabbit polyclonal anti-sera raised against AGE-modified bovine serum albumin (BSA), we found that in animal and human TSEs, one or more lysines at residues 23, 24 and 27 of PrP^{Sc} are covalently modified with advanced glycosylation end products (AGE) (Choi et al., 2004). This modification was seen with all of the TSEs examined: (1) four mouse-adapted scrapie strains (ME7, 139A, 22L and 87V); (2) two hamster-adapted scrapie strains (263K and 139H), and (3) both sporadic CJD and vCJD. Since AGE-modification was observed only in PrP^{Sc}, not in PrP^{C}, antibody against AGE-modified PrP^{Sc} can be used as a specific reagent for PrP^{Sc} immunoassay.

We have applied rabbit polyclonal anti-AGE-BSA Ab (R3) to the LIFIA; if successful, this eliminates the need for PK pretreatment of samples. Anti-AGE-BSA IgG (designated R3) was purified by protein A immunoaffinity chromatography and then labeled with Alexa Fluor 633 for detection or with biotin for capture. First, to validate R3 Ab as a PrP^{Sc}-detecting reagent, Alexa Fluor 633-labeled R3 was added to 300 ul of the assay mixtures containing various biotin-conjugatated anti-PrP monoclonal antibodies (mAbs). For PrP^{C} and PrP^{Sc}-containing samples, 30 ul of brain homogenates from normal and 263K scrapie-infected hamsters, respectively, were used. Although weak positive signals (i.e., S/B ratio above 1.1) were observed for 263K-infected samples using biotin-7A12 or biotin-10E4, there were positive signals from normal hamster brains as well (Fig. 3). Even when negative control samples which do not contain brain homogenates were analyzed, most Ab combinations showed S/B ratios larger than 1.1 (Fig. 3). Inversely, using biotin-R3 Ab and various Alexa Fluor 633-labeled anti-PrP mAbs we tried to detect PrP^{Sc} by LIFIA. As shown in Fig. 4, no PrP^{Sc}-specific positive

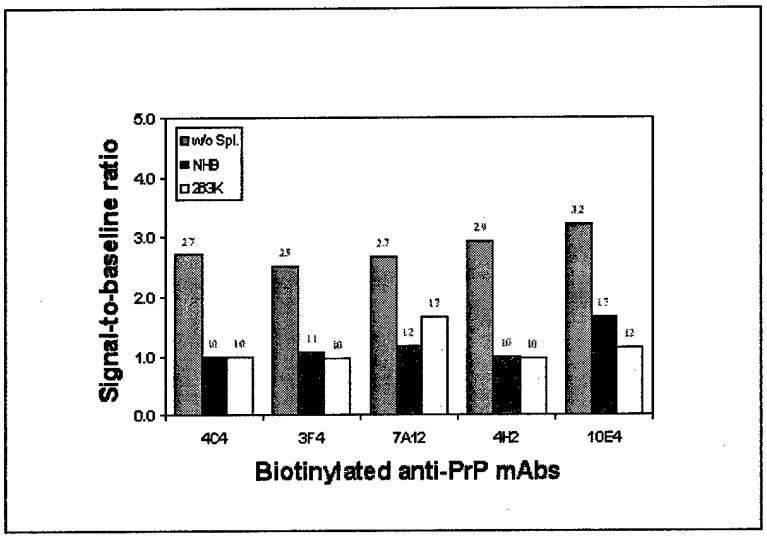

Figure 3. Application of Alexa Fluor 633-labeled anti-AGE-BSA antibody to LIFIA with various biotin-anti-PrP mAbs/streptavidin-beads. Numbers shown above bars represent actual mean values. w/o Spl.: assay mixture without brain samples, NHB: normal hamster brain, 263K: 263K-infected hamster brain.

signals were obtained from various Ab combinations except for Alexa Fluor 633-11H7/biotin-R3. Since 11H7 also showed non-specific reactivity to the magnetic beads in addition to PrP in other tests (data not shown), an S/B ratio of 1.1 from 263K-infected sample does not indicate PrP^{Sc}-specific signal. From these validation tests, it was shown that Alexa Fluor 633-labeled R3 Ab binds to the magnetic beads non-specifically and that R3 Ab may not be suitable for the LIFIA. In our previous study using PrP^{Sc}–enriched fractions, we observed AGE-modification in amino-terminus of PrP^{Sc} from various TSE-affected brains (Choi et al., 2004). Failure to detect PrP^{Sc} by LIFIA using R3 Ab may have resulted from the characteristics of R3 Ab and/or from the presence of other proteins in the analyte. R3 is rabbit polyclonal anti-AGE-BSA Ab and is not specific for

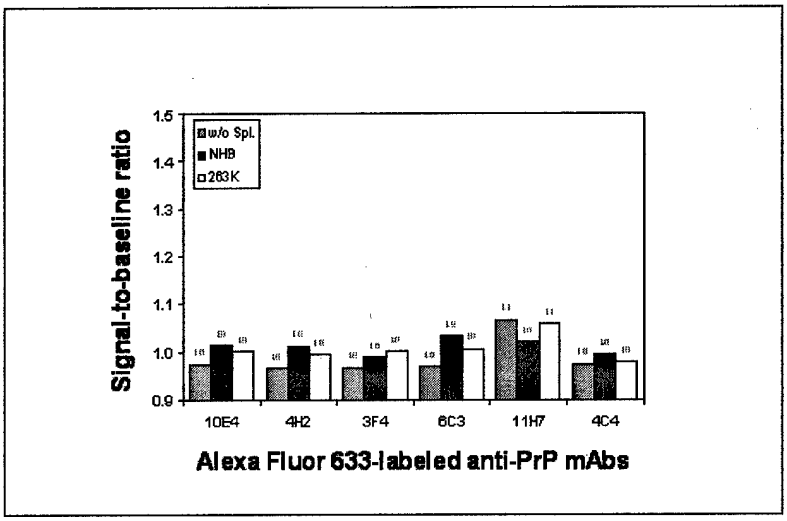

Figure 4. Application of anti-AGE-BSA antibody-conjugated beads to LIFIA with various Alexa Fluor 633-labeled anti-PrP mAbs. Numbers shown above bars represent actual mean values. w/o Spl.: assay mixture without brain samples, NHB: normal hamster brain, 263K: 263K-infected hamster brain.

AGE-modified PrP^{Sc}. This means that many portions of R3 Ab can react with various AGE-modified proteins. It is possible that the binding of certain populations of R3 IgG that are specific for AGE-modified PrP^{Sc} is diminished or interfered with by the co-existence of other proteins during the immunoassay. In order to obtain increased specificity and sensitivity of the LIFIA, the generation of mAbs specific to AGE-modified NH_2-termius of PrP^{Sc} was initiated.

4.2. Generation of mAb against AGE-modified NH_2-termius of PrP^{Sc}

AGE-modified PrP peptide 23-36 (KKRPKPGGWNTGGS) (designated AGE-PrP) was prepared as described previously (Choi et al., 2004). In addition, AGE-modified PrP 23-36 and BSA mixture (designated AGE-PrP-BSA) was prepared as well in order to prevent any failure in inducing the immune reaction against AGE-PrP 23-36 due to its small size as an immunogen. Briefly, PrP peptide or a mixture of BSA and PrP peptide was mixed with D-glucose and dissolved in 0.5 M sodium phosphate buffer (pH 7.4). The solutions were deoxygenated with nitrogen gas, sterilized by ultrafiltration (0.45-μ filter), and then incubated at 37°C for 90 days. After incubation, the samples were dialyzed using three changes of 20 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. The dialyzed samples were lyophilized and then dissolved in sterilized distilled water at 1 mg/ml concentration.

Antigens were prepared for injection by emulsification with adjuvant CytRx (G-5 Titermax Gold, Titermax USA) as described by the manufacturer. Enough emulsified antigens were prepared each time to deliver 200 ul injection per mouse. PrP-knockout mice were immunized every three weeks by subcutaneous and intramuscular injections for a total of nine weeks.

One week after the final boost, the mice were bled retro-orbitally and the antisera were tested for reactivity against the antigen by ELISA (Table1). Sera from mice immunized with AGE-PrP-BSA showed strong reactivity against both immunogens, whereas those from AGE-PrP-immunized mice were much less reactive. The reactivity of polyclonal R3 Ab used as a positive control was similar to the reactivity of sera from mice sera immunized with AGE-PrP. Except for 7A12 (kindly provided by by Dr. Man-Sun Sy at the Institute of Pathology, Case Western University, Cleveland, Ohio, USA)

none of anti-sera showed reactivity against purified 263K PrP^{Sc}. Based on the fact that most of the data from our study (Choi et al., 2004) were obtained using Western blot analysis, we have also tested the reactivity of these sera against various antigens by Western blot.

Table 1. ELISA to test the reactivity of sera taken from immunized mice.

Mouse sera No.	Immunogen		Purified 263K PrP^{Sc}	
	AGE-PrP 23-36	AGE-PrP 23-36 + BSA	PK-	PK+
1	0.137	0.129	0.017	0.017
2	0.140	0.144	0.015	0.022
3	0.128	0.141	0.018	0.022
4	>3.000***	>3.000	0.023	0.040
5	>3.000	>3.000	0.020	0.026
6	0.205	>3.000	0.022	0.033
R3*	0.137	0.292	0.012	0.009
7A12**	0.144	0.118	2.895	0.604

*R3; rabbit polyclonal anti-AGE-BSA Ab, **7A12; anti-PrP mAb, ***>3.000; out of range.

For Western blot, two positive control Abs for either PrP or for AGE-modification were used: anti-PrP 4C4 and polyclonal anti-AGE R3. In Fig. 5, the samples loaded in lanes 1-7 are the same in each of the 8 gels shown (see Fig. 5 legend). Non-PK-treated PrP^{Sc} purified from ME7 and 263K-infected brains (Fig. 5, lanes 1 & 3 in gel # 7), and AGE-modified immunogens (Fig. 5, lanes 5-7 in gel # 7) were easily detected wtih R3 Ab. Ani-PrP 4C4 showed strong reactivity against PrP^{Sc} regardless of PK-treatment. (Fig. 5, lanes 1-4 in gel # 8), but not against AGE-modified peptides or BSA (lanes 5-7 in gel # 8).

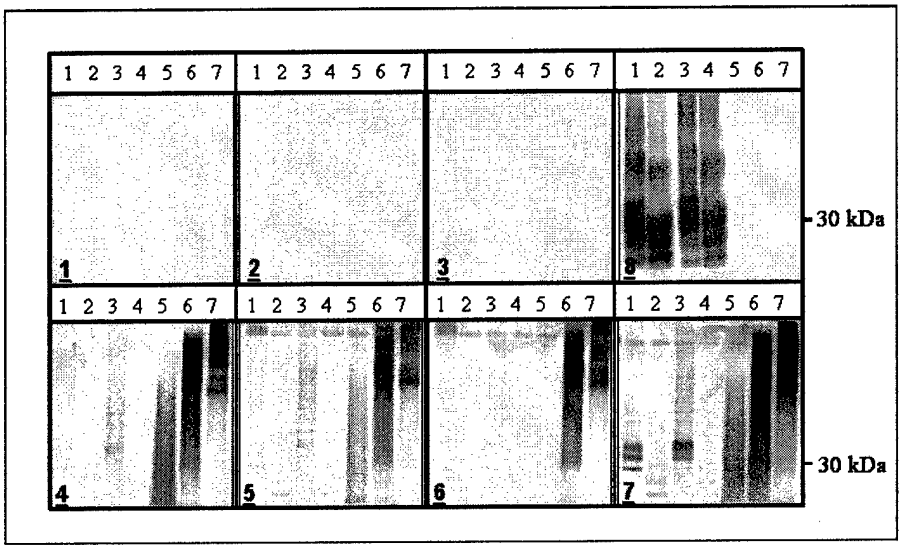

Figure 5. Western blot analysis of sera from PrP knockout mice immunized with AGE-PrP 23-36 or AGE-PrP 23-36-BSA.
Gel # 1-6; sera from mice # 1-6 were tested. Gel # 7 & 8; polyclonal anti-AGE-BSA antibody (# 7) and anti-PrP mAb 4C4 (# 8) were tested.
Lane 1: purified ME7 PrPSc/PK-, 2: purified ME7 PrPSc/PK+, 3: purified 263K PrPSc/PK-, 4: purified 263K PrPSc/PK+, 5: AGE-PrP 23-36, 6: AGE-PrP 23-36-BSA, 7: AGE-BSA

Sera from mice immunized with AGE-PrP were not reactive to AGE-modified immunogens (Fig. 5, gels # 1-3, lanes 5-7 in each gel), whereas sera from mice immunized with AGE-PrP-BSA (Fig. 5, gels # 4 & 5, lanes 5-7 in each gel) reacted with immunogens. Sera from one of the mice (Fig. 5, gel # 6) immunized with AGE-PrP-BSA showed reactivity to AGE-PrP-BSA and AGE-BSA (lanes 6-7), but not to AGE-PrP (lane 5). These Western blot results are consistent with those of ELISA. However immunoreactivity against purified PrP^{Sc} showed different pattern from ELISA. Although there was some difference in the signal intensity between gels, sera from AGE-PrP-BSA-immunized mice (Fig. 5, gels # 4-6) reacted with purified (non-PK-treated) ME7 and 263K PrP^{Sc} (Fig. 5, lanes 1 & 3 in each gel). Sera from mice # 4 and # 5 (Fig. 5, gels # 4 & 5) showed weak reactivity against PK-

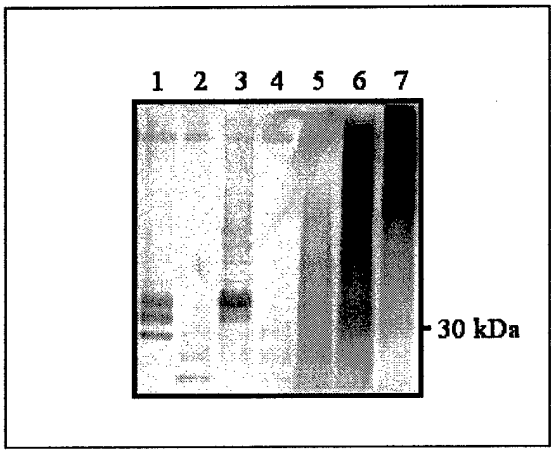

Figure 6. Western blot analysis of sera from mouse # 5 immunized with AGE-PrP 23-36-BSA prior to sacrifice for fusion.
Lane 1: purified ME7 PrPSc/PK-, 2: purified ME7 PrPSc/PK+, 3: purified 263K PrPSc/PK-, 4: purified 263K PrPSc/PK+, 5: AGE-PrP 23-36, 6: AGE-PrP 23-36-BSA, 7: AGE-BSA

treated PrP^{Sc} as well (Fig. 5, lanes 2 & 4 in each gel). Based on these results, mouse # 5 was chosen for fusion with myeloma. At present, generation of a hybridoma cell line secreting anti-AGE-PrP mAb is ongoing in our laboratory. Prior to sacrificing mouse # 5 for fusion, the blood was collected by eye-bleeding and serum was tested again by Western blot to confirm its reactivity against PrP^{Sc} or AGE-PrP (Fig. 6). Anti-serum showed stronger immunoreactivity to all tested samples (PrP^{Sc} and AGE-modified immunogens), especially to non-PK-treated PrP^{Sc} (Fig. 6, lanes 1-3).

4.3. Application of anti-DNA OCD-4 antibody to the immunoassay.

As an alternative approach to exclude the need for PK pretreatment during the assay, we have applied other PrP^{Sc}-specific reagents to the LIFIA. Zou et al. (2003) reported that anti-DNA Ab OCD-4 and gene 5 protein (g5p), which is a single-stranded DNA binding protein, identified PrP^{Sc} in an immunocapture assay. These DNA-binding reagents have been shown to capture PrP^{Sc}, not PrP^{C}.

Human and mouse OCD-4s, and g5p were kindly provide by Dr. Shu Chen at the Institute of Pathology, Case Western University, Cleveland, Ohio, USA. Prior to applying these DNA-binding reagents to the LIFIA, we have tested their immunocapture activity to PrP from various TSE-affected animals. OCD-4 and g5p (100 ug) were conjugated to 7×10^8 tosyl-activated magnetic beads (M-280 Dynabeads) in 1 ml of PBS at 37°C for 20 h. The conjugated beads were incubated with 0.1% BSA in PBS to block nonspecific binding. For immunoprecipitation, brain homogenates (10%, w/v) of various animals with or without TSE were prepared at 4°C in lysis buffer (100 mM NaCl/10 mM EDTA/0.5% NP-40/0.5% sodium deoxycholate/10 mM Tris_HCl, pH 7.5) containing a mixture of protease inhibitors (Roche Applied Science), followed by centrifugation at 3,000 $\times g$ for 10 min at 4°C to remove debris. Immunoprecipitation (IP) was then performed by using the homogenates (50 ul each) and OCD4- or g5p-conjugated beads (10 ug of mAb/6×10^7 beads) in 1 ml of lysis buffer. After incubation for 3 hrs at room temperature, the beads were washed with PBS (pH 7.5) containing 2% Tween 20 and 2% NP-40 and bound material was eluted by heating at 99°C for 5 min in 2× SDS-PAGE sample buffer. As a positive control for PrP immunoprecipitation, 4C4-beads were

included. The eluted materials were separated on 12% Tris-glycine gel and analyzed by Western blot.

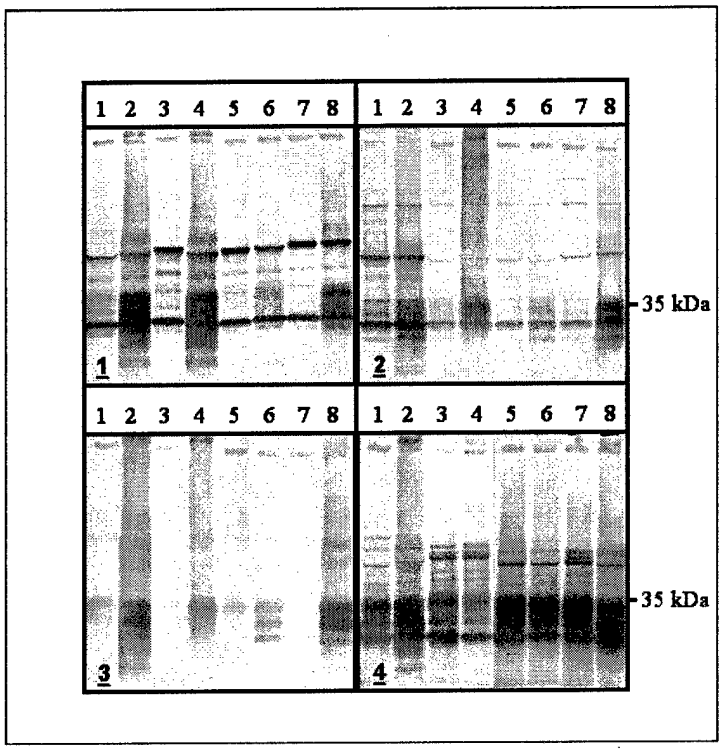

Fig. 7. Western blot (WB) analysis of PrP immunoprecipitated (IP) by using DNA binding reagent.
Gel 1: IP with human OCD-4 and WB with 4C4, 2: IP with mouse OCD-4 and WB with 4C4, 3: IP with g5p and WB with 4C4, 4: IP with 4Cr and WB with 4C4.
Lane 1: normal mouse brain, 2: ME7-infected mouse brain, 3: normal hamster brain, 4: 263K-infected hamster brain, 5: normal mule deer brain, 6: CWD-infected mule deer, 7: normal sheep brain, 8: scrapie-infected sheep brain.

Without PK treatement, PrP^{C} and PrP^{Sc} in brain homogenates from normal and scrapie-infected TSE animals were immunoprecipitated with 4C4-beads (Fig. 7, gel # 4). Although some faint signals from normal brain samples were detected, strong PrP bands were detected from all of TSE-affected animals when human and mouse OCD-4- , and g4p-coated beads were used (Fig. 7, gel # 1, 2, 3, respectively): ME7 in mouse, 263K in hamster, CWD in mule deer, scrapie in sheep in lanes 2, 4, 6, and 8, respectively, in each gel.

Next, we tested the applicability of these beads to the LIFIA. The OCD-4-coated beads were added to the assay mixture containing non-PK-treated brain homogenates from normal and scrapie-infected animals. After 3 hrs incubation at room temperature, various Alexa Fluor 633-labeled anti-PrP mAbs (2.5 ug/ml reaction mixture) were added to each mixture and further incubated for 1 hr. Finally, the beads were washed 3 times with washing buffer (PBS, pH 7.5/2% Tween 20/2% NP-40) and the bound materials were eluted with elution buffer (0.1 M glycine, pH 2.8). Using LIF spectrofluometer,

fluorescence intensity of each sample was measured for 50 sec with a 10 sec integration time.

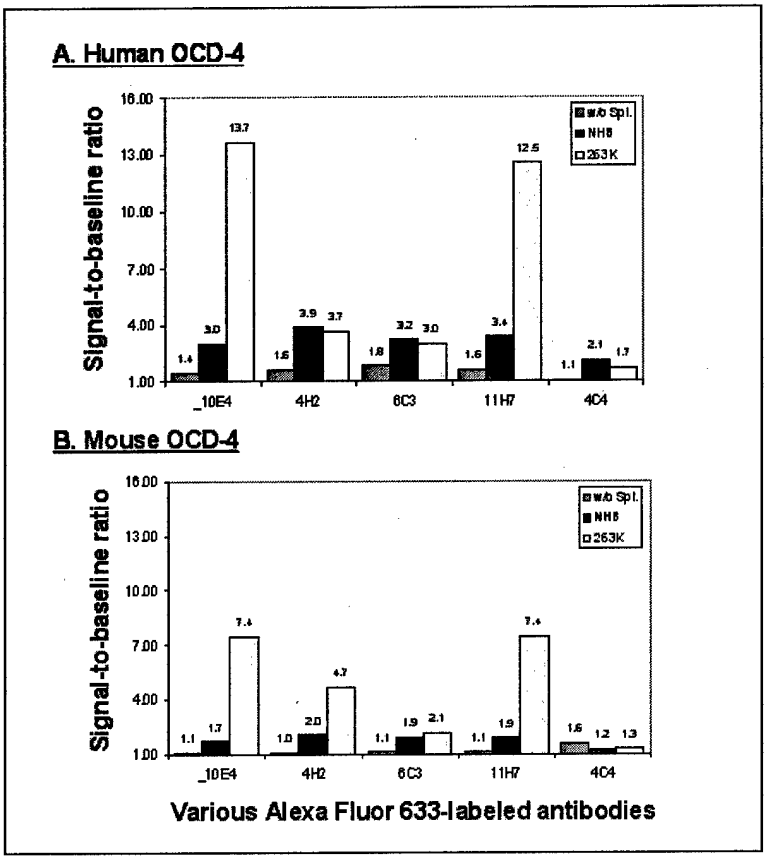

Fig. 8. Application of OCD-4-beads to the LIFIA with various Alexa Fluor 633-labeled anti-PrP mAbs. Numbers shown above bars represent actual mean values.
w/o Spl.: assay mixture without brain samples, NHB: normal hamster brain, 263K: 263K-infected hamster brain.

Fig. 8 shows the results from LIFIA using human and mouse OCD-4 beads with various Alexa Fluor 633-labeled anti-PrP mAbs. The S/B ratios for Alexa Fluor 633-labeled 10E4 and 11H7 were significantly higher for 263K vs. NHB and no sample (w/o Spl.) using both human and mouth OCD-4. There was a smaller S/B ratio difference with Alexa Fluor 633-labeled 4H2, but only with mouse OCD-4. The other 3 Abs showed no significant differences in S/B ratio among the three preparations. We have analyzed the S/B ratios from Alexa-10E4 and 11H7 (Fig. 9). Positive signals with S/B ratio larger than 1.1 were detected in the absence of brain homogenates in the assay mixture. Furthermore, moderate S/B ratios from normal hamster brains were detected as well. These results indicate that there was some non-specific binding of human and mouse OCD-4 beads with anti-PrP mAb. However, it was clear that the S/B ratios from scrapie-infected hamster brains were much higher than from normal hamster brains regardless of the kind of beads used. These results suggest that if the difference in the S/B ratios between normal and infected animals can be seen consistently from many analyzed samples, it could be an alternative

criterion to identify TSE-positive samples. Currently, we are examining this possibility by analyzing more samples. In addition, we are testing various experimental conditions to reduce the non-specific binding of OCD-4 with anti-PrP mAbs.

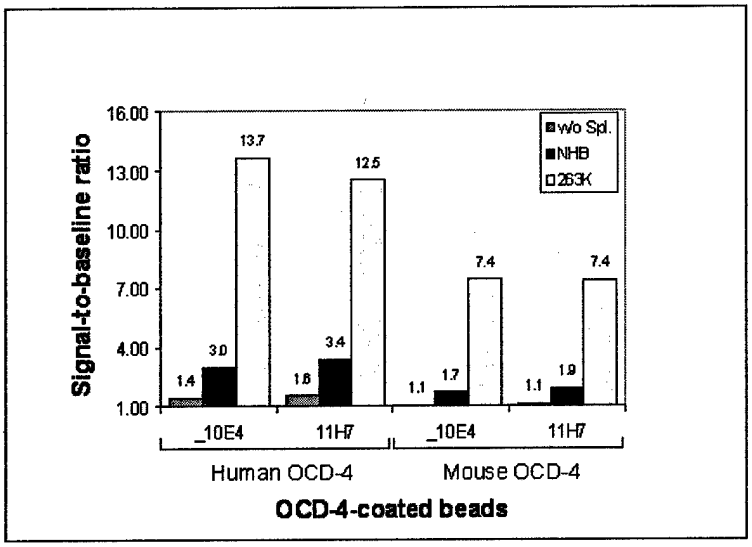

Fig. 9. Application of OCD-4-beads to the LIFIA with Alexa Fluor 633-labeled 10E4 and 11H7. Numbers shown above bars represent actual mean values.
w/o Spl.: assay mixture without brain samples, NHB: normal hamster brain, 263K: 263K-infected hamster brain.

4.4. Comparison of Alexa Fluor 633 and Alexa Fluor 635 dyes.

Brightness of the fluorophores is one of the critical factors determining the sensitivity of the LIFIA. So far we have been using Alexa Fluor 633 (Molecular Probes, USA) because its maximum excitation wavelength at 633 nm is compatible with the light source (Helium-Neon laser) of our LIF spectrofluorometer. Recently, a new commercial dye, Alexa Fluor 635, became available. This dye has the same excitation and emission wavelengths (excitation 633 nm/emission 647 nm) as those of Alexa Fluor 633, however, Alexa Fluor 635 has no self-

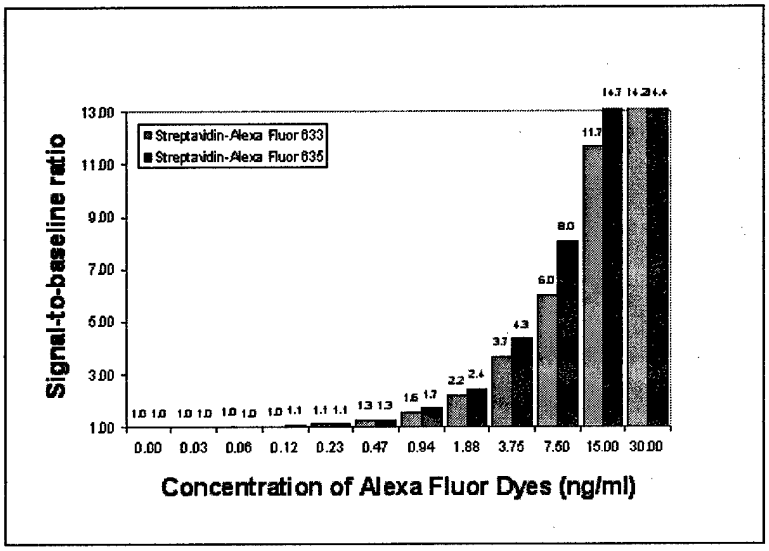

Figure 10. Comparison of the brightness of Alexa Fluor 633 and Alexa Fluor 635 dyes. Fluorescence from various dilutions of streptavidin-conjugated Alexa Fluor dyes were read using laser-induced fluorescence spectrofluorometer.

quenching of fluorescence and higher molecular extinction coefficient ($\varepsilon \cong 200{,}000\ cm^{-1}M^{-1}$) than Alexa Fluor 633. We have examined whether Alexa Fluor 635 would be better than Alexa Fluor 633 in terms of the brightness by comparing the fluorescence intensity of various dilutions of these two dyes. Streptavidin-conjugated Alexa Fluor 633 (2.2 mole dye/streptavidin) and Alexa Fluor 635 (3.9 mole dye/streptavidin) were serially diluted in PBS and fluorescence was read for 10 sec integration time. As shown in Fig. 10, fluorescence intensity from Alexa Fluor 635 over various concentrations was higher than the intensity of Alexa Fluor 633. However, there was not a marked difference in the lowest amounts of the dye that could be detected by the instrument (0.23 ng/ml of Alexa Fluor 633 vs. 0.12 ng of Alexa Fluor 635). This difference may be a function of the degree of labeling of the two dyes (2.2 mole/streptavidin for Alexa Fluor 633 vs. 3.9 mole dye/streptavidin for Alexa Fluor 635) rather than due to the characteristics of each dye.

4.5. Immunoprecipitation of plasma PrP using 4C4-coated magnetic beads.

As a pilot experiment to test the applicability of LIFIA to the detection of PrP in blood samples, we performed immunoprecipitation of PrP from normal and 263K-infected hamster plasma using 4C4-coated magnetic beads. Plasma was isolated from heparinized blood of normal and infected hamsters. Some plasma samples were dialyzed against saline and treated with Affi-Gel Blue affinity gel (Bio-Rad, USA) to remove albmin, and then treated with or without PK. One ml of processed or non-processed plasma were diluted in 9 ml of immunoprecipitation buffer (Tris-Cl, pH 8.0/1% sodium deoxycholate/1% NP-40/150 mM NaCl/1 mM EDTA/ 0.2% SDS) (Kim et al., 2004) and heated at 99°C for 5 min. Fifty ul of 4C4-coated beads were added to the mixture and incubated for 1.5 hrs at room temperature. As a positive control for immunoprecipitation of PrP, 1 ml of 10% 263K hamster brain homogenates treated with or without PK was used. After extensive washing with PBS containing 0.1% Tween-20, bound materials were eluted from the beads by heating at 99°C for 5 min in 2× SDS-PAGE sample buffer. Eluted samples were separated on 12% SDS-PAGE and analyzed by Western blot using biotin-7A12 as a primary Ab.

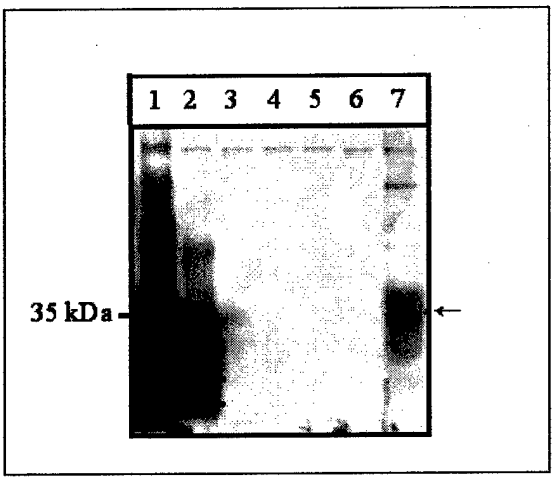

Fig. 11. Detection of plasma PrP by Western blot followed by immunoprecipitaton with 4C4-coated beads. Primary antibody, 7A12 was used for blot.
Lane1: 263K brain homogenates/PK-, 2: 263K brain homogenates/PK+, 3: Normal hamster plasma/PK-/dialyzed & Affi-Gel-treated, 4: Normal hamster plasma/PK+/dialyzed & Affi-Gel-treated, 5: 263K hamster plasma/PK-/dialyzed & Affi-Gel-treated, 6: 263K hamster plasma/PK+/dialyzed & Affi-Gel-treated, 7: Normal hamster plasma/PK-

From 263K-infected brain as positive control, large amounts of PrP^{Sc} were obtained (Fig. 11, lanes 1 & 2). However, no signals were detected from the processed- normal or 263K-infected hamster plasma (Fig. 11, lanes 3-6), whereas PrP^{C} with molecular weight ranging from 27-36 kDa was detected from non-processed plasma from normal hamster (Fig. 11, lane 7). Although more extensive experiments are needed to optimize the condition for the detection of PrP in blood, our result is promising because PrP^{C} in plasma could be detected by immunoprecipitation followed by Western blot. To our knowledge, there are no reports on the direct detection of blood PrP^{C} by Western blot. This means that the concentration step included in the LIFIA procedure would be advantageous in the successful detection of blood infectivity or PrP^{Sc}.

5. Hardware Improvements to Existing LIF Spectrometer and Development of Fiber Based LIF Spectrometer

Los Alamos National Laboratory suspended operation on 16 July 2004 pending a review of operations and safety and work resumed in late September. Thus, this report covers progress during an abbreviated work period.

5.1. Modifications to Existing LIF Spectrometer

The goal of this activity is to increase the sensitivity of the spectrometric system such that it can measure samples with substantially higher dilution than it's present capability. Since at present, the capability is in the femtomolar region for certain dyes,

we are pushing the limits of instrumentation. The spectrometer is equipped with a thermoelectrically cooled, back-illuminated, thinned Hamamatsu CCD array which has maximum sensitivity in the red portion of the spectrum but has at least 80 percent of it's peak sensitivity in the green. The red He-Ne laser that is used for the source is rated at 10 mW output. The greatest leverage that we could achieve in increasing the sensitivity of the instrument is to change the laser to a green solid state device. The green excitation wavelength is usually superior to a red one both for fluorescence and for Raman scattering. In the case of fluorescence, most dyes have a larger absorption cross section in the green than in the red leading to a higher fluorescence yield. In the case of Raman scattering, the cross section is proportional to the third power of the inverse ratio of the wavelengths. This leads to a 60% increase in signal. Thus, we purchased a small solid state laser based upon a diode-pumped, frequency doubled Nd:YAG crystal. We bought two lasers, one having an output of 5 mW and the other having an output of 25 mW. It was our initial intention to install the latter device in the IBR spectrometer. Our Laboratory restart procedure prevented us from doing this as the 25 mW laser is a class 3B device which we were not yet permitted to use. Thus, we installed the five mW device

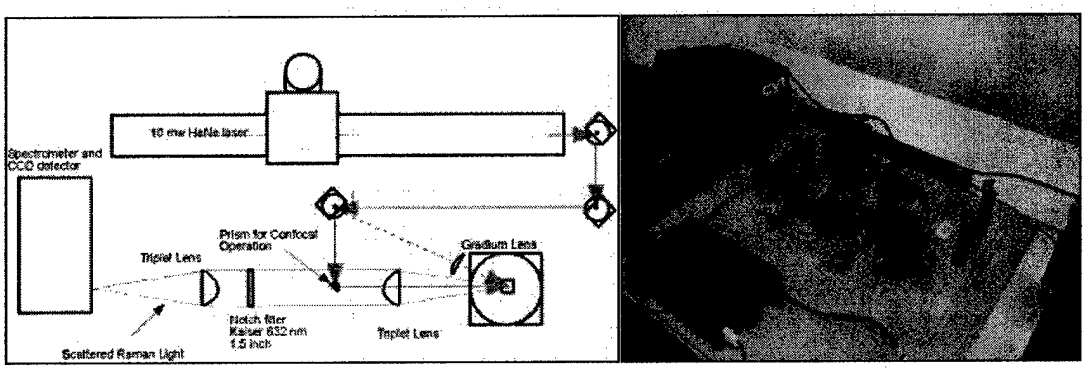

Figure 12. Laser-induced fluorescence spectrometer, optical design and system.

in the IBR spectrometer. Of course, we also installed the appropriate holographic notch filter to reject the 532nm fundamental wavelength as well as a safety shutter that is interlocked with the spectrometer cover. After a thorough checkout and realignment of the system, our technician was able to achieve an increase in sensitivity of at least 30%.

5.2. Current Status of Existing LIF System

Subsequent measurements showed a decrease in sensitivity as compared to previous fluorescence determinations. We have analyzed the data and have come to the following conclusions. After performing an end-to-end review of the spectrometer design, including the laser source, the focusing optics, the transfer optics on the detector side of the sample as well as the spectrometer/detector pair, we believe that additional tuning of the optics is necessary. [Please refer to Fig. 12] Specifically, the most likely cause for a decrease in sensitivity to have occurred is that correlation between the short focal length microscope objective, on the excitation side of the optics, and Gradium lenses that focuses the fluorescence on the spectrometer entrance is off. If this is correct, it would be impossible for the two triplet lenses to relay the fluorescence signal to the spectrometer. We are developing the safety document that will get us management approval to correct this, and we will upgrade the laser to a higher out put head at the same time.

5.3. Fiber Optic Coupled Microcapillary Detection System

Efficiency of excitation and efficiency of collection of fluorescence light are the aims of this task. To this end, we have designed an assembly consisting of a microcapillary of 100 μl capacity (using a Drummond microcapillary as a prototype sample holder) that is surrounded by four linear arrays of fiber optical elements. The capillary tube is removable from the assembly, and the design is easily modified to accommodate small volume capillaries. It can use a flow-thru or a statically filled tube as well as one with the excitation traveling along the length of the tube. The arrangement is shown in Fig. 13. The geometric advantage of such an apparatus is immediately obvious. Essentially all fluorescence light that is emitted from the sample is collected by the surrounding arrays of fiber. In addition to this, the design can be adapted to detect up to four channels of fluorescence by splitting out each of the four detector bundles of fibers and incorporating additional detectors (PMTs or avalanche diodes) with the appropriate filters. This detection scheme also has the advantage that the only component that is required to be in the laboratory is the sample receiver (a machined aluminum block and a few feet of optical fiber). Current optical fiber technology is such that signal drop with

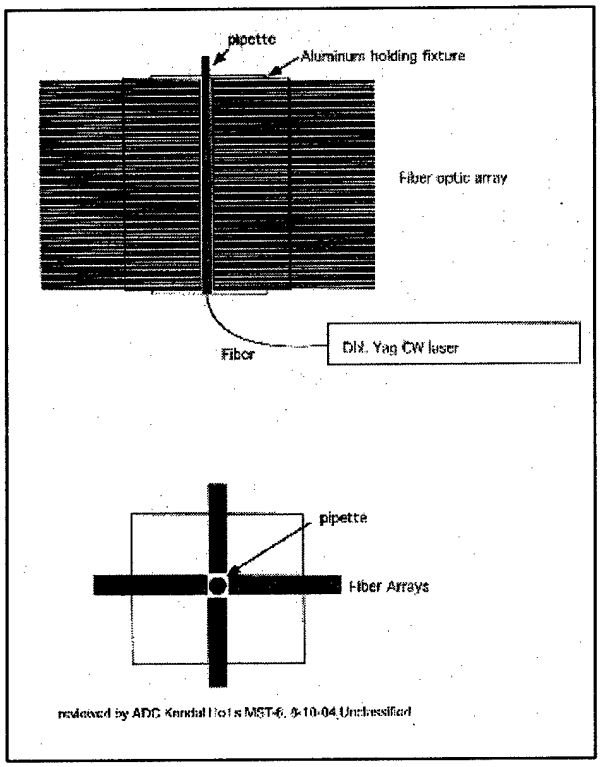

Fig. 13. Fiber-based design

distance is of the order of a dB per kilometer, so the detector itself and the data acquisition system for the instrument do not need to be collocated with the receiver for the the sample. This solves the problem of having to commit an instrument to exclusive use when deploying it in BSL3 facility.

5.4. Current Status of Fiber Based System

We have been in consultation with Polymicro Technologies Inc. and have finalized a design for the linear fiber arrays to be used in the detection head. These arrays will be assembled in the next few weeks, and a prototype head will be machine to accept them.

5.5. Ultraviolet laser

A laser source for excitation of dyes is by far the most desirable of all light sources. This is because it has the highest spectral brightness (Watts/cm^2 steradian nm) of any light source. This is due to the laser's small beam divergence, often less than one milliradian and its exceedingly small spectral bandwidth, usually less than 10^{-4} nm. The former quantity permits the laser to be focused into a very small spot in the sample to be interrogated, providing the highest possible excitation intensity. Thus, we purchased a small frequency doubled Nd:YAG laser whose output at 532 nm can be frequency doubled to 266 nm using a nonlinear optical apparatus that is part of our optics laboratory. The actual driving laser is no larger than a laser pointer and the doubling crystal has a volume of only a few cubic centimeters. The hard ultraviolet supplied by such a source should be much more effective in excitation of highly efficient dyes, such as the Rhodamines which have large absorption features in there, and the use of quantum dots.

Key Research Accomplishments

1. The LIFIA test successfully distinguished normal from CWD retropharyngeal lymph node preparations.
2. NaPTA treatment is an effective method for concentrating PrP preparations prior to LIFIA.
3. Antibodies to AGE and DNA provide promising additional probes for LIFIA analyses.
4. Anti-PrP mAb-coated beads were used to concentrate PrP, thus improving the potential for detection.

Reportable Outcomes

N/A.

Conclusions

The work during the first year of this contract has provided important advancement in our capacity to effectively pursue a specific, sensitive PrP^{Sc} assay in easily obtainable biological fluids. Improvements in both the biological and hardware areas have been made; this will further our efforts toward the goal of a fluorescent immunoassay system using a laser-induced fluorescence spectrofluorometer.

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Appendices

N/A